Dietary -linolenic acid inhibits arterial thrombus formation, tissue factor expression, and platelet activation

Holy, E W ; Forestier, M ; Richter, E K ; Akhmedov, A ; Leiber, F ; Camici, G G ; Mocharla, P ; Lüscher, T F ; Beer, J H ; Tanner, F C

Abstract: OBJECTIVE: Plant-derived -linolenic acid (ALA) may constitute an attractive cardioprotective alternative to fish-derived n-3 fatty acids. However, the effect of dietary ALA on arterial thrombus formation remains unknown. METHODS AND RESULTS: Male C57Bl/6 mice were fed a high-ALA or low-ALA diet for 2 weeks. Arterial thrombus formation was delayed in mice fed a high-ALA diet compared with those on a low-ALA diet (n=7; P<0.005). Dietary ALA impaired platelet aggregation to collagen and thrombin (n=5; P<0.005) and decreased p38 mitogen-activated protein kinase activation in platelets. Dietary ALA impaired arterial tissue factor (TF) expression, TF activity, and nuclear factor- B activity (n=7; P<0.05); plasma clotting times and plasma thrombin generation did not differ (n=5; P=not significant). In cultured human vascular smooth muscle and endothelial cells, ALA inhibited TF expression and activity (n=4; P<0.01). Inhibition of TF expression occurred at the transcriptional level via the mitogen-activated protein kinase p38 in smooth muscle cells and p38, extracellular signal-regulated kinases 1 and 2, and c-Jun N-terminal kinases 1 and 2 in endothelial cells. CONCLUSIONS: ALA impairs arterial thrombus formation, TF expression, and platelet activation and thereby represents an attractive nutritional intervention with direct dual antithrombotic effects.

DOI: https://doi.org/10.1161/ATVBAHA.111.226118

Dietary Alpha-Linolenic Acid Inhibits Arterial Thrombus Formation, Tissue Factor Expression, and Platelet Activation

Erik W. Holy, MD[1,2,3], Marc Forestier, PhD[4], Eva K. Richter, Msc[5], Alexander Akhmedov, PhD[1,2], Florian Leiber, PhD[5], Giovanni G. Camici, PhD[1,2], Pavani Mocharla, Msc[1,2], Thomas F. Lüscher, MD,[1,2,3], Jürg H. Beer, MD[4] and Felix C. Tanner, MD[1,2,3]

[1]Cardiovascular Research, Institute of Physiology, University of Zurich, Switzerland
[2]Center for Integrative Human Physiology, University of Zurich, Switzerland
[3]Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland
[4]Department of Medicine, Cantonal Hospital of Baden, Switzerland
[5]Department of Agricultural and Food Science, Federal Institute of Technology Zurich, Switzerland

Abstract

Objective. Plant-derived *alpha*-linolenic acid (ALA) may constitute an attractive cardioprotective alternative to fish-derived *n*-3 fatty acids. However, the effect of dietary ALA on arterial thrombus formation remains unknown.

Methods and Results. Male C57Bl/6 mice were fed a high ALA or low ALA diet for 2 weeks. Arterial thrombus formation was delayed in mice fed a high ALA diet as compared to those on a low ALA diet (n=7; $p<0.005$). Dietary ALA impaired platelet aggregation to collagen and thrombin (n=5; $p<0.005$) and decreased p38 MAP kinase activation in platelets. Dietary ALA impaired arterial tissue factor (TF) expression, TF activity and NFκB activity (n=7; $p<0.05$); plasma clotting times and plasma thrombin generation did not differ (n=5; p=NS). In cultured human vascular smooth muscle and endothelial cells ALA inhibited TF expression and activity (n=4; $p<0.01$). Inhibition of TF expression occurred at the transcriptional level via the MAP kinase p38 in smooth muscle cells and p38, ERK1/2, and JNK1/2 in endothelial cells.

Conclusions. ALA impairs arterial thrombus formation, TF expression, and platelet activation, and thereby represents an attractive nutritional intervention with direct dual anti-thrombotic effects.

Key words: alpha-linolenic acid; thrombosis; platelets; tissue factor, MAP kinases

Arterial thrombosis is the critical step in the development of acute vascular syndromes.[1,2,3] Circulating platelets are activated by interaction with subendothelial collagen at the site of an injury.[4] In parallel, membrane bound tissue factor (TF) expressed on vascular cells acts as a receptor for activated factor VII (VIIa).[5,6] The TF/VIIa complex triggers the coagulation cascade and the formation of activated factor X (Xa) ultimately resulting in thrombin formation, which in turn cleaves protease-activated receptors on the platelet surface boosting platelet activation and clot formation[4]. The critical role of platelets and TF in the pathogenesis of acute coronary syndromes has indeed been well documented.[7,8,9]

Experimental and epidemiological studies have extensively characterized the cardioprotective and anti-thrombotic effects of fish-derived dietary long chain *n*-3 fatty acids eicosapentaenoic acid (C20:5 *n*-3, EPA) and docosahexaenoic acid (C22:6 *n*-3, DHA).[10,11,12,13] Consistent with these observations, an increased risk for developing cardiovascular disease was identified in populations with low intake of EPA and DHA, prompting the American Heart Association (AHA) to modify their nutrition guidelines.[14]

ALA is an essential *n*-3 FA found at high concentrations in vegetable oils, in particular flaxseed oil, where it accounts for 50% of the total FA content. Following oral intake, ALA is partially converted into EPA and DHA. The rate of conversion of ALA into long chain *n*-3 fatty acids ranges from 0.05 to 10%.[15] In recent epidemiological studies, ALA consumption has been inversely associated with the incidence of myocardial infarction, sudden cardiac death, and coronary artery calcification.[16-20] Despite these promising data, the precise biological mechanisms mediating the cardioprotective effects of ALA remain barely understood. In particular, it remains controversial whether the rather poor conversion of ALA to EPA and DHA can account for the beneficial effects of ALA or whether ALA exerts direct biological

effects.[21,22]

Since increasing evidence suggests that ALA can serve as a cardioprotective nutritional supplement, this study addresses the question whether dietary ALA inhibits thrombus formation *in vivo* and analyzes the mechanisms involved.

Materials and Methods

For the detailed Materials and Methods please see the supplemental Materials and Methods (available online at http://atvb.ahajournals. org).

ALA diet and carotid artery thrombosis model

C57BL/6 mice were fed a 0.21% w/w cholesterol diet containing either a high ALA (7.3%) or low ALA concentration (0.03%, supplemental table 1).[23] Photochemical injury model was performed after 2 weeks of treatment.

Immunohistochemistry

Thrombus composition was analysed in paraffin fixed sections from occluded carotid arteries. Sections were stained for TF, fibrin, and the platelet marker CD41.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT)

Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500g; 4°C; 15 minutes). PT and aPTT were assessed using the START 4 analyzer (Diagnostica Stago, France). [24]

Thrombin generation

Plasma thrombin generation was monitored by automated calibrated thrombography and the results analysed using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).[25,26]

Platelet aggregation

Platelet aggregation was studied by whole blood impedance aggregometry (Chrono-Log, Havertown, PA, USA).

Fatty acid profile in aortic tissue

FA profiles of the pooled tissues were analyzed by gas chromatography as described.[27] Measurements were performed in duplicate and the mean value displayed.

Cell culture

Human aortic vascular smooth muscle cells (VSMC; Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC; Clonetics) were cultured as described.[24] Protein expression was determined by Western blot analysis and RNA by real-time polymerase chain reaction (PCR).

Tissue factor activity

Factor Xa generation on the surface of VSMC, HAEC, or in tissues was analyzed using a colorimetric assay (American Diagnostica Inc, ACTICHROME).

TF promoter activity

TF promoter activity was measured as described.[28] A minimal TF promoter (-227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene and a recombinant adenoviral vector was constructed. HAEC were transduced with the vector Ad5/hTF/Luc (100 pfu/cell) for 1 hour. An adenoviral vector without reporter gene (VQAd/Empty) was used as a negative control. After transduction, HAEC were grown in medium (10% FBS) for 24 hours and then serum-starved for 24 hours prior to TNF-α stimulation with or without ALA (30 μM) pretreatment. Firefly luciferase

activity was determined by luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized to the protein concentration in the lysates.

Nuclear extracts and NFκB activity

Nuclear extracts were obtained from HAEC or mouse aorta using a nuclear extraction kit (Active Motif, Carlsbad, USA) and NFκB activity measured using a TransAM NFκB p65 kit (Active Motif).

Statistics

Data are presented as mean±SEM. Statistics were performed using the GraphPad prism 4.0 Software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using 2-tailed unpaired Student´s t-test or one-way ANOVA as appropriate. A value of $p<0.05$ was considered significant.

Results

Dietary ALA inhibits arterial thrombosis

C57BL/6 mice were fed for 2 weeks a 0.21% cholesterol diet containing either 7.3% w/w ALA (high ALA, treated group) or 0.03% w/w ALA (low ALA, control group). No difference in body weight was observed after 2 weeks of diet (n=7; *p*=NS; data not shown). Aorta from animals fed a high ALA diet showed markedly increased ALA levels (14.9% of total FA content; supplemental table 2) as compared to tissue from control animals (0.14% of total FA content; supplemental table 2). In contrast, EPA and DHA levels did not differ between the two groups. Total cholesterol levels were not altered (control: 2.27±0.17 mmol/l; low ALA: 1.95±0.21 mmol/l; high ALA: 2.29±0.30 mmol/l; n=8; *p*=NS).

Mice on control diet developed carotid artery thrombosis within a mean occlusion time of 40±4.2 minutes, while mice on high ALA diet occluded after 68±6 minutes (n=7; $p<0.005$; figure 1A). Initial blood flow did not differ between the two groups (n=7; *p*=NS). Intravenous administration of an inhibitory anti-TF antibody before the onset of photochemical injury reduced the difference in occlusion times between control and high ALA diet (n≥7; *p*=NS; figure 1B). Plasma clotting times (PT and aPTT) were similar in the two groups (PT: 11.1±0.2 seconds [low ALA] versus 11.5±0.4 seconds [high ALA]; n=7; *p*=NS; aPTT: 20.3±0.8 seconds [low ALA] versus 21.1±0.7 seconds [high ALA]; n=7; *p*=NS). Thrombin generation (endogenous thrombin potential [ETP] and time to peak [TTP]) did not differ between the groups (ETP: 747±94 nM/minute [low ALA] versus 818±80 nM/minute [high ALA]; n=5; *p*=NS; TTP: 6.2±0.7 minutes [low ALA] versus 4.8±0.5 minutes [high ALA]; n=5; *p*=NS). Further no difference in peak thrombin values (104.4±28.7 nM [low ALA] versus 118.8±22.9 nM [high ALA]; n=5; *p*=NS) nor in lag times (2.9±1.36 minutes

[low ALA] versus 2.1±1.3 minutes [high ALA]; n=5; *p*=NS) was observed between the groups.

To analyse differences in thrombus composition, sections from occluded carotid arteries were stained for TF, fibrin, and platelets. Although thrombi were occlusive in both groups, TF staining of in the vascular media was lower in the high ALA group as compared to the control group (figure 1C; n=4). In contrast, fibrin staining was similar in thrombi from both groups. Analysis of platelet content by immunhistochemistry for CD41 demonstrated a reduced platelet content in thrombi in the ALA treated group as compared to control (figure 1D; n=4).

Dietary ALA inhibits platelet aggregation

Thrombin induced platelet aggregation (figure 2A-D) was inhibited in mice fed a high ALA diet as compared to control platelets (maximal aggregation: n=5, $p<0.005$, figure 2B; area under the curve: n=5; $p<0.005$, figure 2C; lag time: n=5, $p<0.05$, figure 2D). When aggregation was induced by collagen, a similar inhibition was observed (maximal aggregation: n=5, $p<0.005$, figure 2F; area under the curve: n=5, $p<0.05$, figure 2G; lag time: n=5, $p=0.14$, figure 2H). Platelet number did not differ significantly between the groups ($1062\pm46x10^3$/µL [low ALA] versus $911\pm5x10^3$/µL [high ALA]; n=5; *p*=NS).

Treatment of human platelets with ALA (30 µM) for 30 minutes *ex vivo* exerted a similar inhibition of thrombin and collagen induced platelet aggregation (n=5; supplemental table 3A-B). ALA (30 µM) impaired collagen and thrombin induced p38 phosphorylation (n=3; $p<0.01$ versus collagen or thrombin alone; supplemental figure 1A-B).

Dietary ALA inhibits TF expression and NFκB activation

TF activity in carotid arteries was markedly decreased in mice fed a high ALA diet as compared to controls (n=7; p<0.005, figure 3A). Inhibition of TF with a specific antibody confirmed that factor Xa generation was TF dependent (figure 3B). This decrease in TF activity was paralleled by an impaired TF mRNA expression in aortic tissue (n=7; p<0.01; figure 3C). In contrast, expression of tissue factor pathway inhibitor (TFPI) mRNA did not differ (ΔΔCt: 0.03±0.04 low ALA group versus 0.03±0.04 high ALA; n=5; p=NS). NFκB p65 DNA binding affinity was measured in nuclear extracts from aortic tissue. NFκB activity was impaired in mice fed a high ALA diet as compared to controls (OD 490 nm: 0.97±0.02 [low ALA] versus 0.70±0.09 [high ALA]; n=7; p<0.05, figure 3D).

ALA inhibits TF protein expression in VSMC

Treatment with ALA (30 μM) for 24 hours reduced TF expression in VSMC by 46% (n=4; *p*<0.05) as compared to control (figure 4A). Real time PCR analysis confirmed that ALA inhibited TF expression in VSMC at the transcriptional level (n=4; p<0.005; figure 4B). In line with this observation, ALA decreased TF activity in VSMC (n=4; *p*<0.001; figure 4C). Experiments performed with an inhibitory anti-TF antibody confirmed that factor Xa generation in VSMC was TF dependent (figure 4D). TF expression was neither affected by the *n*-6 FA alpha-linoleic acid (LA) nor the saturated FA stearic acid (SA) (n=4; *p*=NS; data not shown). No cytotoxic effects of ALA, LA, or SA in VSMC were observed for any of the concentrations (data not shown).

ALA inhibits p38 phosphorylation and NFkB activity in VSMC

Analysis of the MAP kinase phosphorylation demonstrated that treatment of VSMC with ALA for 24 hours resulted in a significant decrease in p38 activation

(49±14% inhibition; n=4; p<0.05; figure 5A). In contrast, activation of JNK and ERK remained unaffected. Blockade of the MAP kinase p38 with SB203580 (10 μM) inhibited TF expression in VSMC (n=4; p<0.01; figure 5B). Treatment with ALA (30 μM) reduced NFκB p65 DNA binding affinity in nuclear extracts from VSMC (n=4; *p*<0.05; figure 5C).

ALA inhibits TNF-α induced TF mRNA expression via MAP kinase and ASK1 in HAEC

HAEC were treated with ALA or vehicle for 1 hour and then stimulated with TNF-α (5 ng/mL) for 4 hours. ALA inhibited TNF-α induced endothelial TF expression in a concentration dependent manner with a maximal effect at 30 μM (86% inhibition; n=5; *p*<0.001 versus TNF-α alone; figure 6A). In line with this observation, ALA decreased TF activity in HAEC (51% inhibition versus TNF-α alone; n=4; *p*<0.001; figure 6B).

Real-time PCR analysis demonstrated that ALA (30 μM) inhibited TNF-α induced endothelial TF mRNA expression (n=4; *p*<0.001; Figure 6C). This decrease in TF mRNA was paralleled by an impaired activation of the MAP kinases JNK, p38, and ERK (28%, 62%, and 55% inhibition versus TNF-α alone, respectively; n=4; *p*<0.05; figure 6D). Consistent with this pattern of MAP kinase inhibition, ALA impaired activity of the MAPKKK ASK1 (n=3; *p*<0.005 versus TNF-α alone; figure 6E). ALA (30 μM) inhibited TNF-α induced TF promoter activation in HAEC by 47% (n=4; *p*<0.05, figure 6F). ALA (30 μM) blunted TNF-α induced IκB-α degradation (n=3; *p*<0.05). This effect was paralleled by reduced DNA binding activity of the NFκB subunit p65 (54% inhibition versus TNF-α alone, n=4; *p*<0.01). To confirm the involvement of NFκB in TNF-α induced endothelial TF expression, the effect of different NFκB inhibitors was assessed. Both BAY 11-7082 (5 μM) and PDTC (10

μM) inhibited TNF-α induced TF expression (-66% and -47% versus TNF-α alone, respectively; n=4; $p<0.01$).

Discussion

Variations of the dietary *n*-3/*n*-6 ratio have been reported to affect the susceptibility to thrombosis in atherosclerotic mice.[29] However, it remains unknown whether ALA can exert such an effect and whether if influence thrombosis directly or via other PUFA.

The present study demonstrates that dietary supplementation with ALA for 2 weeks impaired arterial thrombus formation, platelet activation, TF activity, and NFκB activity in mice in vivo. The ALA rich diet increased tissue levels of ALA, while there was no significant change in tissue levels of the long chain *n*-3 FA EPA and DHA. Consistent with these findings, ALA by itself impaired platelet aggregation and TF induction in different human primary cells cultures. This effect occurred at the transcriptional level involving inhibition of MAP kinase phosphorylation, NFκB activation, and promoter activation.

The ALA concentrations used for experiments in this study are in a clinically relevant range. Indeed, baseline plasma levels of ALA have been reported to range from 17-19 μM, whereas a daily oral ingestion of 3 g ALA leads to ALA plasma levels of 32±17 μM and is well tolerated[15]. Dietary supplementation of ALA for 2 weeks was sufficient to markedly increase the ALA levels in the aortic wall, whereas EPA and DHA levels did not differ from the control group. The lack of a measurable conversion to long chain *n*-3 FA is in line with previous epidemiological studies demonstrating that dietary ALA poorly correlates with EPA and DHA levels in adipose tissue, erythrocytes or plasma[16]. Even more important, the *in vivo* effects of dietary ALA

observed in the present study were mimicked by exogenous ALA in both human platelets and vascular cells *ex vivo*, where hepatic conversion of ALA to EPA and DHA does not occur. Taken together, these experiments indicate that the anti-thrombotic effects of ALA *in vivo* did not depend on the conversion of ALA to long chain *n*-3 FA and provide strong evidence for a direct biological effect of ALA.

To exclude possible effects of other FA, control experiments were performed with the *n*-6 FA linoleic acid as well as the saturated FA stearic acid. Neither of these FA altered TF expression, supporting the interpretation that the biological effects of the high ALA diet on TF and thrombosis occur due to the increased ALA levels.

Dietary ALA supplementation significantly delayed arterial thrombus formation triggered by photochemical injury *in vivo*. Since this effect was paralleled by a reduced platelet activation and a diminished arterial TF activity, ALA impairs thrombus formation by a dual action on both critical events involved in arterial thrombosis following vascular injury. In order to determine the relative contribution of platelets and TF to thrombus formation, an inhibitory anti-TF antibody was applied. Treatment with this antibody reduced the difference in occlusion times between the two groups, suggesting that the inhibitory effect of ALA on TF exerts a major effect on thrombus formation, while its effect on pletelets contributes to inhibition of thrombosis. These results support previous observations demonstrating that arterial thrombosis is primarly driven by TF derived from the vessel wall in this model.[30,31] TF is barely expressed under basal conditions in endothelial cells, while it is constitutively expressed in VSMC.[32] Immunohistochemical analysis confirmed that TF expression in the arterial tunica media was decreased in carotid arteries derived from the ALA treated group. Inhibition of TF was confirmed *in vivo*, since the ALA rich diet decreased TF expression and NFkB activity in arterial lysates. Consistent with these in vivo observation, treatment of VSMC with ALA for 24 hours decreased basal TF

expression. This effect was mediated at the transcriptional level via the MAP kinase p38. Pharmacological inhibition of p38 confirmed the crucial role of p38 activation in constitutive TF expression in VSMC. In line with p38 inhibition as well as with previous data obtained in monocytes and macrophages ALA also reduced NFkB activity in VSMC.[33,34] Hence, direct inhibition of TF in VSMC via p38 and NFkB seems to play a major role in ALA's effect on arterial thrombus formation.

In order to study whether ALA also affects inducible TF expression, additional experiments were performed in cytokine activated endothelial cells. TNF-α induced endothelial TF expression is mediated through activation of MAP kinases leading to activation of transcription factors such as AP-1 and EGR-1, whereas the IκB kinase pathway promotes NFκB activation.[35,36] ASK1 is a redox regulated kinase playing an essential role in stress induced activation of MAP kinases, thus triggering various cellular processes including inflammatory responses and TF expression.[32,37] In endothelial cells, TNF-α has been shown to activate ASK1 as well as MAP kinases.[6,37] Treatment with ALA significantly reduced phosphorylation of the MAP kinases p38, JNK, and ERK in endothelial cells. Consistent with the inhibition of MAP kinase phosphorylation, ALA also prevented activation of ASK1. Thus, it is likely that ASK1 represents a common upstream target of ALA disrupting the MAP kinase signalling pathway in endothelial cells.

Immunohistochemical analysis of CD41 (platelet glycoprotein IIb) expression demonstrated that thrombi contained less platelets in the ALA treated group as compared to the control group. Platelet aggregation was analyzed using collagen and thrombin as agonists, since they play a dominant role in platelet activation following vascular injury.[3] ALA impaired both collagen and thrombin induced platelet aggregation, indicating that ALA inhibits glycoprotein VI and Ib-V-IX as well as protease-activated receptor mediated platelet activation; hence, ALA does not

interfere with activation of a single pathway. Since the MAP kinase p38 plays a crucial role in collagen as well as thrombin induced platelet activation and adhesion, the effect of ALA on p38 phosphorylation in isolated platelets was investigated.[38,39] ALA decreased collagen and thrombin induced p38 activation, an effect that may well account for the reduced aggregation observed in ALA treated platelets. Taken together, these observations may provide an explanation for the remaining difference in occlusion times observed between the two groups after treatment with an inhibitory anti-TF antibody.

In summary, this study provides solid evidence for a potent dual anti-thrombotic effect of an ALA rich diet by reducing platelet activation and impairing vascular TF expression. These effects were reproduced in a mouse model as well as in human primary cell cultures and were not related to an increase in EPA and DHA levels as confirmed by our data. Hence, this study generates pathophysiological evidence for direct anti-thrombotic effects of dietary ALA supplementation. Since the limited availability or unfavourable geographic conditions restrict the access to *n-3* FA from marine origin in many countries, plant-derived ALA might therefore represent an attractive cardioprotective alternative. However, keeping in mind previous clinical setbacks with experimentally promising nutritional compounds such as vitamin E, there is a great need for placebo-controlled randomized large-scale clinical trials to confirm the long-term anti-thrombotic potential of dietary supplemented ALA.

Funding Sources

This work was supported by a grant from the Swiss National Science Foundation (grant no. 3200B0-113328/1 to FCT, grant no. 3100-068118.02/1 to TFL, grant no. 310030-130500 to GGC and grant no. 310030-127422 to JHB).

References

1. Fuster V, Badimon L, Badimon J, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. N Engl J Med. 1992; 326:310-318.
2. Fuster V. Lewis A. Conner Memorial Lecture: Mechanisms leading to myocardial infarction: insights from studies of vascular biology. Circulation. 1994; 90:2126-2146.
3. Furie B, Furie BC. Mechanisms of thrombus formation. N Engl J Med. 2008; 359:938-949.
4. Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. J Clin Invest. 2000; 105:699-701.
5. Mackman N. The role of tissue factor and factor VIIa in hemostasis. Anesth Analg. 2009; 108:1447-1452.
6. Steffel J, Lüscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation. 2006; 113:722-31.
7. Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation. 1995;

92:657-671.

8. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. Circulation 1996; 94:1226-1232.

9. Meadows TA, Bhatt DL. Clinical aspects of platelet inhibitors and thrombus formation. Circ Res. 2006; 100:1261-1275.

10. Lee JH, O'Keefe JH, Lavie CJ, Marchioli R, Harris WS. Omega-3 fatty acids for cardioprotection. Mayo Clin. Proc. 2008; 83:324–332.

11. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation. 2002; 106:2747–2757.

12. Lavie CL, Milani RV, Mehra MR, Ventura HO. Omega-3 polyunsaturated fatty acids and cardiovascular diseases—a fish tale with growing credibility. J. Am. Coll. Cardiol. 2009; 54:585–594.

13. Andriamampandry MD, Leray C, Freund M, Cazenave JP, Gachet C. Antithrombotic effects of (n-3) polyunsaturated fatty acids in rat models of arterial and venous thrombosis. Thromb Res. 1999; 93:9-16.

14. Gidding SS, Lichtenstein AH, Faith MS, Karpyn A, Mennella JA, Popkin B, Rowe J, Van Horn L, Whitsel L. Implementing American Heart Association Pediatric and Adult Nutrition Guidelines: A Scientific Statement From the American Heart Association Nutrition Committee of the Council on Nutrition, Physical Activity and Metabolism, Council on Cardiovascular Disease in the Young, Council on Arteriosclerosis, Thrombosis and Vascular Biology, Council on Cardiovascular Nursing, Council on Epidemiology and Prevention, and Council for High Blood Pressure Research. Circulation. 2009; 119:1161-1175.

15. Harper CR, Edwards MJ, DeFilipis AP, Jacobson TA. Flaxseed Oil Increases the Plasma Concentrations of Cardioprotective (n-3) Fatty Acids in Humans. J Nutr. 2006; 136:83-87.
16. Burdge GC, Calder PC. α-linolenic acid metabolism in adult humans: the effects of gender and age on conversion to longer-chain polyunsaturated fatty acids. Eur J Lipid Sci Technol. 2005; 107:426-439.
17. Campos H, Baylin A, and Walter C. Willett. α-Linolenic Acid and Risk of Nonfatal Acute Myocardial Infarction. Circulation. 2008; 118:339-345.
18. Albert CM, Oh K, Whang W, Manson JE, Chae CU, Stampfer MJ, Willett WC, and Hu FB. Dietary α-Linolenic Acid Intake and Risk of Sudden Cardiac Death and Coronary Heart Disease. Circulation. 2005; 112:3232-3238.
19. Djoussé L, Arnett DK, Carr JJ, Eckfeldt JH, Hopkins PN, Province MA, Ellison RC. Dietary Linolenic Acid Is Inversely Associated With Calcified Atherosclerotic Plaque in the Coronary Arteries: The National Heart, Lung, and Blood Institute Family Heart Study. Circulation. 2005; 111:2921–2926
20. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, Guidollet J, Touboul P, Delaye J. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet. 1994; 343: 1454–1459.
21. Harris WS. Cardiovascular Risk and α-Linolenic Acid: Can Costa Rica Clarify? Circulation. 2008; 118:323-324.
22. Harris WS. Alpha-Linolenic Acid: A Gift From the Land? Circulation. 2005; 111:2872-2874.
23. Matter CM, Lohmann C, Tanner FC, Holy EW, Richter EK, Leiber F, Lüscher TF, Forestier M, Beer JH. Alpha-linolenic acid diminishes atherothrombosis, reduces red cell arachidonic acid and endothelial tissue factor expression. J Thromb Haemost 2009; 7, Suppl. 2: Abstract OC-TU-052.

24. Breitenstein A, Stämpfli SF, Camici GG, Akhmedov A, Ha HR, Follath F, Bogdanova A, Lüscher TF, Tanner FC. Amiodarone inhibits arterial thrombus formation and tissue factor translation. Arterioscler Thromb Vasc Biol. 2008; 28:2231-2238.

25. Tchaikovski SN, VAN Vlijmen BJ, Rosing J, Tans G. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. J Thromb Haemost. 2007; 5:2079-2086.

26. Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Béguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. Thromb Haemost. 2000; 83:589-591.

27. Richter EK, Shawish KA, Scheeder M, Colombani PC. Trans fatty acid content of selected Swiss food: The TransSwissPilotStudy. J. Food Compos. Anal. 2009; 22: 479-484.

28. Holy EW, Akhmedov A, Lüscher TF, Tanner FC. Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells. J Mol Cell Cardiol. 2009; 46:234-240.

29. Yamashita T, Oda E, Sano T, Yamashita T, Ijiru Y, Giddings JC, Yamamoto J. Varying the ratio of dietary n-6/n-3 polyunsaturated fatty acid alters the tendency to thrombosis and progress of atherosclerosis in apoE-/- LDLR-/- double knockout mouse. Thromb Res. 2005;116:393-401.

30. Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N, Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood. 2005; 105:192-198.

31. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. Wang L, Miller C, Swarthout RF, Rao M, Mackman N, Taubman MB. Blood. 2009;113:705-713.

32. Tilley R, Mackman N. Tissue factor in hemostasis and thrombosis. Semin Thromb Hemost. 2006; 32:5-10.

33. Ren J, Chung SH. Anti-inflammatory effect of alpha-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-kappaB and mitogen-activated protein kinase pathways. J Agric Food Chem. 2007; 55:5073-5080.

34. Perez-Martinez P, Lopez-Miranda J, Blanco-Colio L, Bellido C, Jimenez Y, Moreno JA, Delgado-Lista J, Egido J, Perez-Jimenez F. The chronic intake of a Mediterranean diet enriched in virgin olive oil, decreases nuclear transcription factor kappaB activation in peripheral blood mononuclear cells from healthy men. Atherosclerosis. 2007; 194:141-146.

35. Luyendyk JP, Piper JD, Tencati M, Reddy KV, Holscher T, Zhang R, Luchoomun J, Chen X, Min W, Kunsch C, Mackman N. A novel class of antioxidants inhibit LPS induction of tissue factor by selective inhibition of the activation of ASK1 and MAP kinases. Arterioscler Thromb Vasc Biol. 2007; 27:1857-1863.

36. Breitenstein A, Camici GG, Tanner FC. Tissue factor: beyond coagulation in the cardiovascular system. Clin Sci. 2009; 118:159-172.

37. Zhang R, He X, Liu W, Lu M, Hsieh JT, Min W. AIP1 mediates TNF-alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3.J Clin Invest. 2003;111:1933-1943.

38. Kramer RM, Roberts EF, Strifler BA, Johnstone EM. Thrombin induces activation of p38 MAP kinase in human platelets. J Biol Chem. 1995; 270: 27395-27398.

39. Mazharian A, Roger S, Maurice P, Berrou E, Popoff MR, Hoylaerts MF, Fauvel-Lafeve F, Bonnefoy A, Bryckaert M. Differential involvement of ERK2 and p38 in platelet adhesion to collagen. J Biol Chem. 2005; 280: 26002–26010.

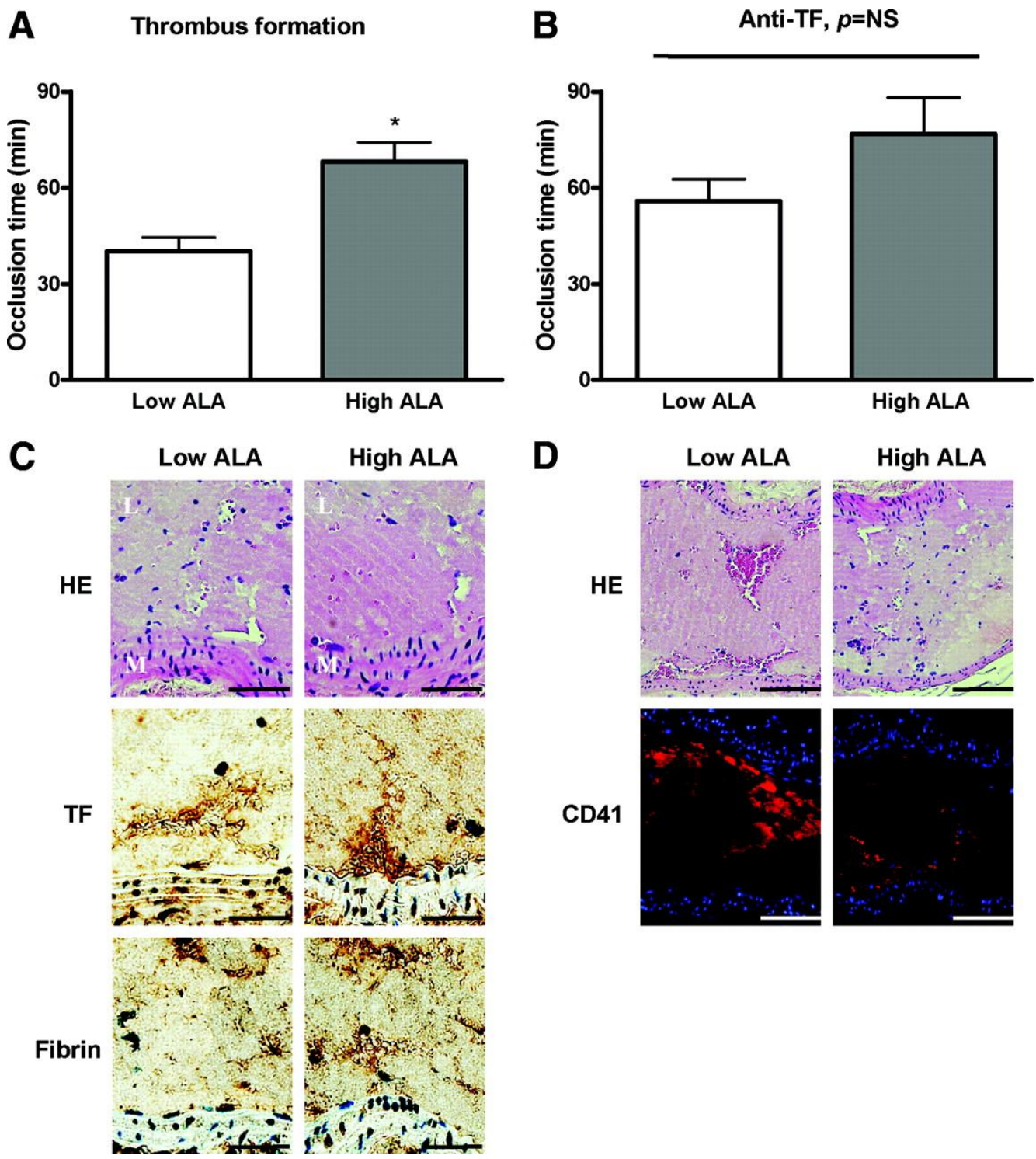

A
Thrombus formation
Occlusion time (min)
90
60
30
0
*
Low ALA
High ALA
B
Anti-TF, p=NS
Occlusion time (min)
90
60
30
0
Low ALA
High ALA
C
Low ALA
High ALA
HE
TF
Fibrin
L
M
D
Low ALA
High ALA
HE
CD41

Figure 1

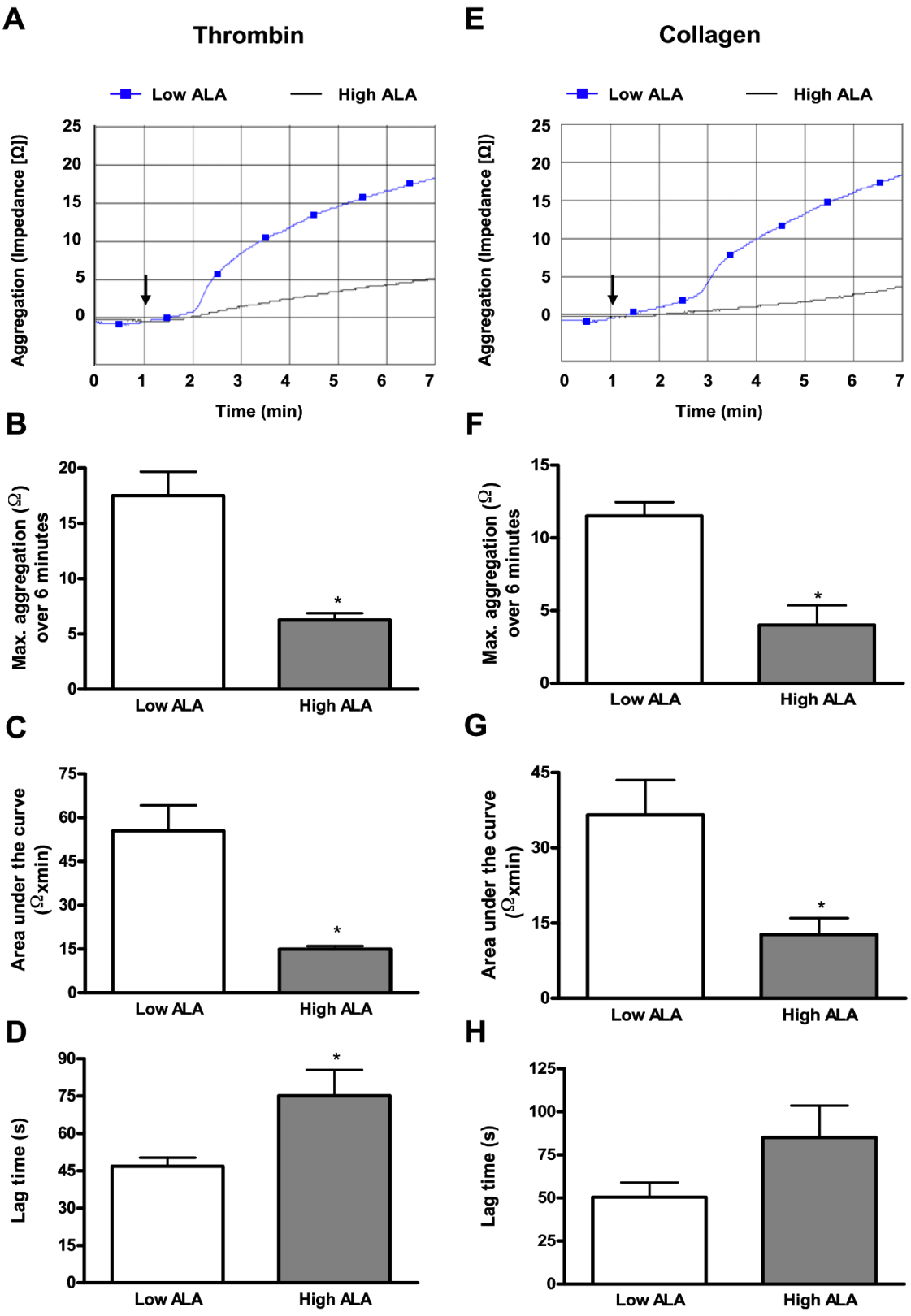

A
Thrombin
Low ALA
High ALA
Aggregation (Impedance [Ω])
Time (min)
E
Collagen
Low ALA
High ALA
Aggregation (Impedance [Ω])
Time (min)
B
Max. aggregation (Ω) over 6 minutes
Low ALA
High ALA
F
Max. aggregation (Ω) over 6 minutes
Low ALA
High ALA
C
Area under the curve (Ωxmin)
Low ALA
High ALA
G
Area under the curve (Ωxmin)
Low ALA
High ALA
D
Lag time (s)
Low ALA
High ALA
H
Lag time (s)
Low ALA
High ALA

Figure 2

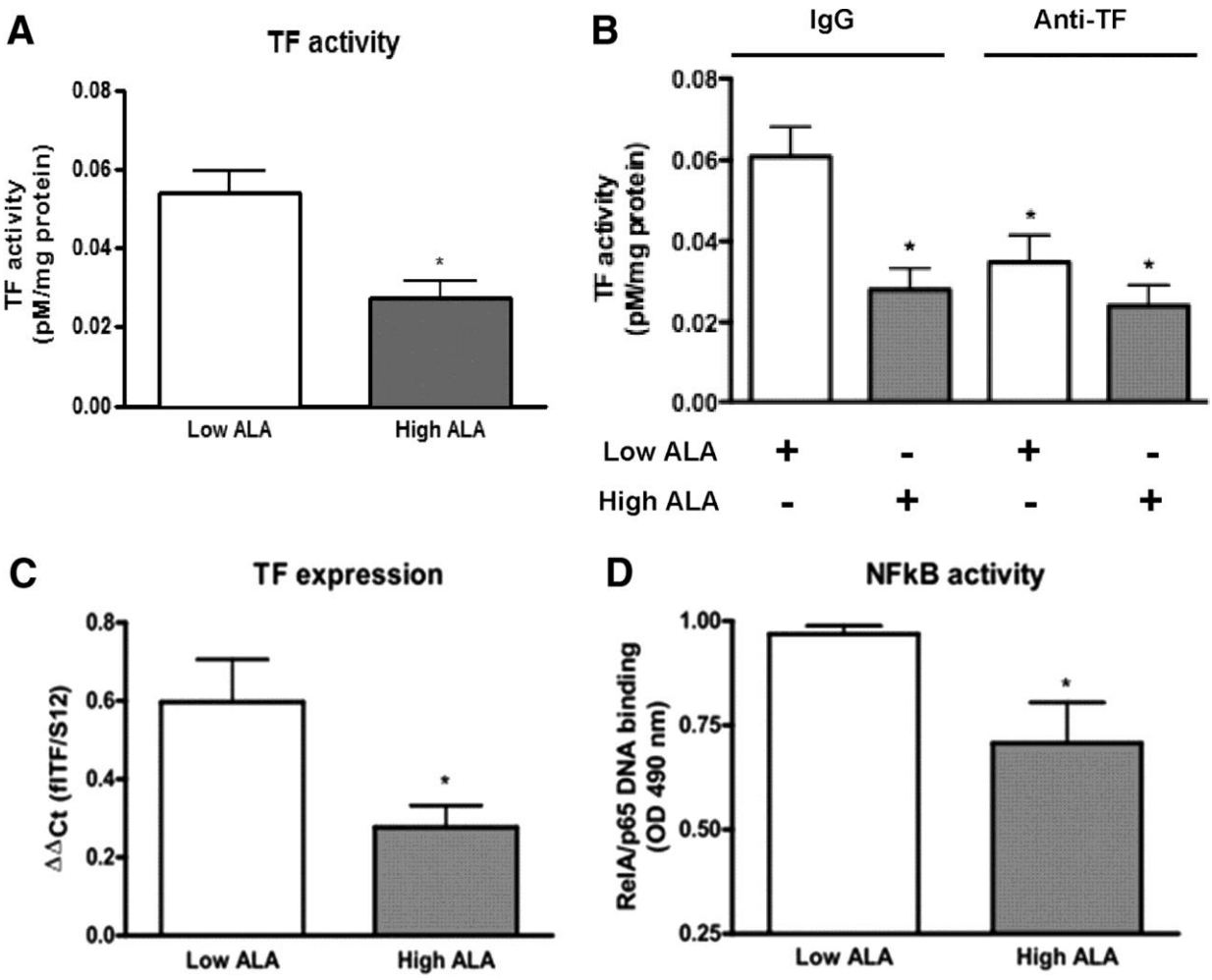

A
TF activity
TF activity (pM/mg protein)
0.08
0.06
0.04
0.02
0.00
*
Low ALA
High ALA
B
IgG
Anti-TF
TF activity (pM/mg protein)
0.08
0.06
0.04
0.02
0.00
*
*
*
Low ALA + - + -
High ALA - + - +
C
TF expression
ΔΔCt (flTF/S12)
0.8
0.6
0.4
0.2
0.0
*
Low ALA
High ALA
D
NFkB activity
RelA/p65 DNA binding (OD 490 nm)
1.00
0.75
0.50
0.25
*
Low ALA
High ALA

Figure 3

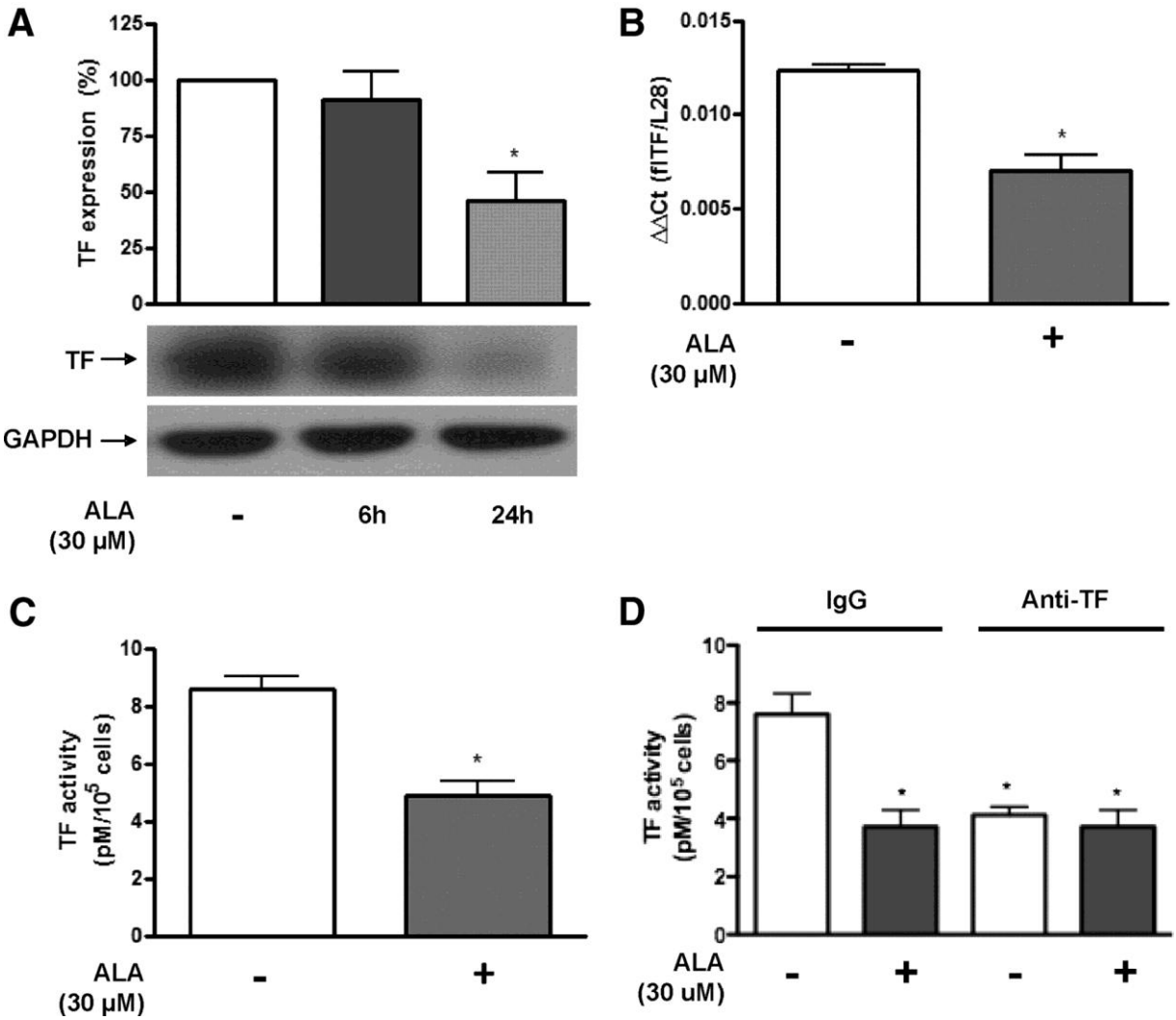

A
TF expression (%)
125
100
75
50
25
0
*
TF
GAPDH
ALA
(30 μM)
-
6h
24h
B
ΔΔCt (fITF/L28)
0.015
0.010
0.005
0.000
*
ALA
(30 μM)
-
+
C
TF activity
(pM/10^5 cells)
10
8
6
4
2
0
*
ALA
(30 μM)
-
+
D
IgG
Anti-TF
TF activity
(pM/10^5 cells)
10
8
6
4
2
0
*
*
*
ALA
(30 uM)
-
+
-
+

Figure 4

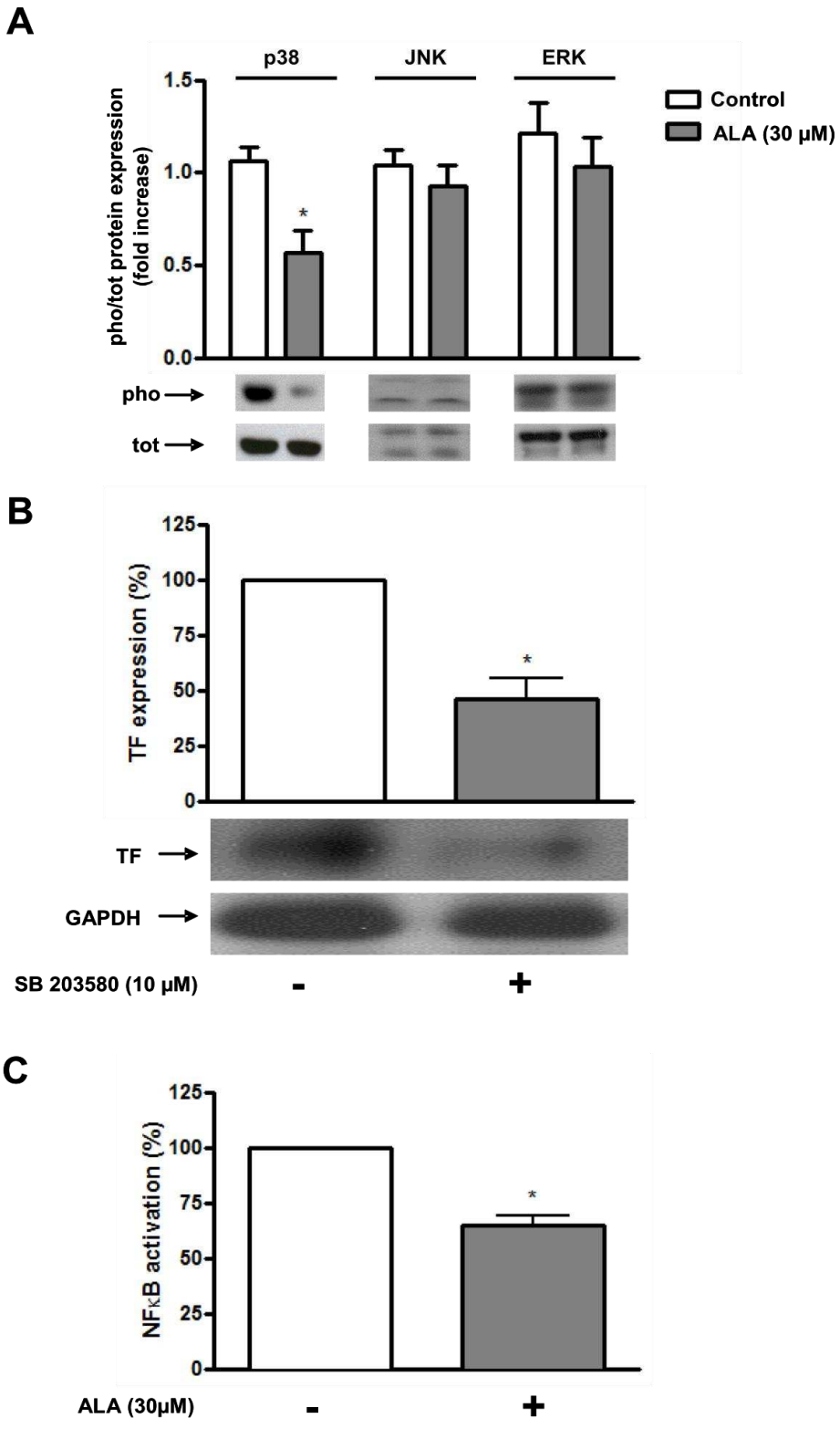

A
p38
JNK
ERK
Control
ALA (30 μM)
pho/tot protein expression (fold increase)
1.5
1.0
0.5
0.0
*
pho
tot
B
TF expression (%)
125
100
75
50
25
0
*
TF
GAPDH
SB 203580 (10 μM)
-
+
C
NFκB activation (%)
125
100
75
50
25
0
*
ALA (30μM)
-
+

Figure 5

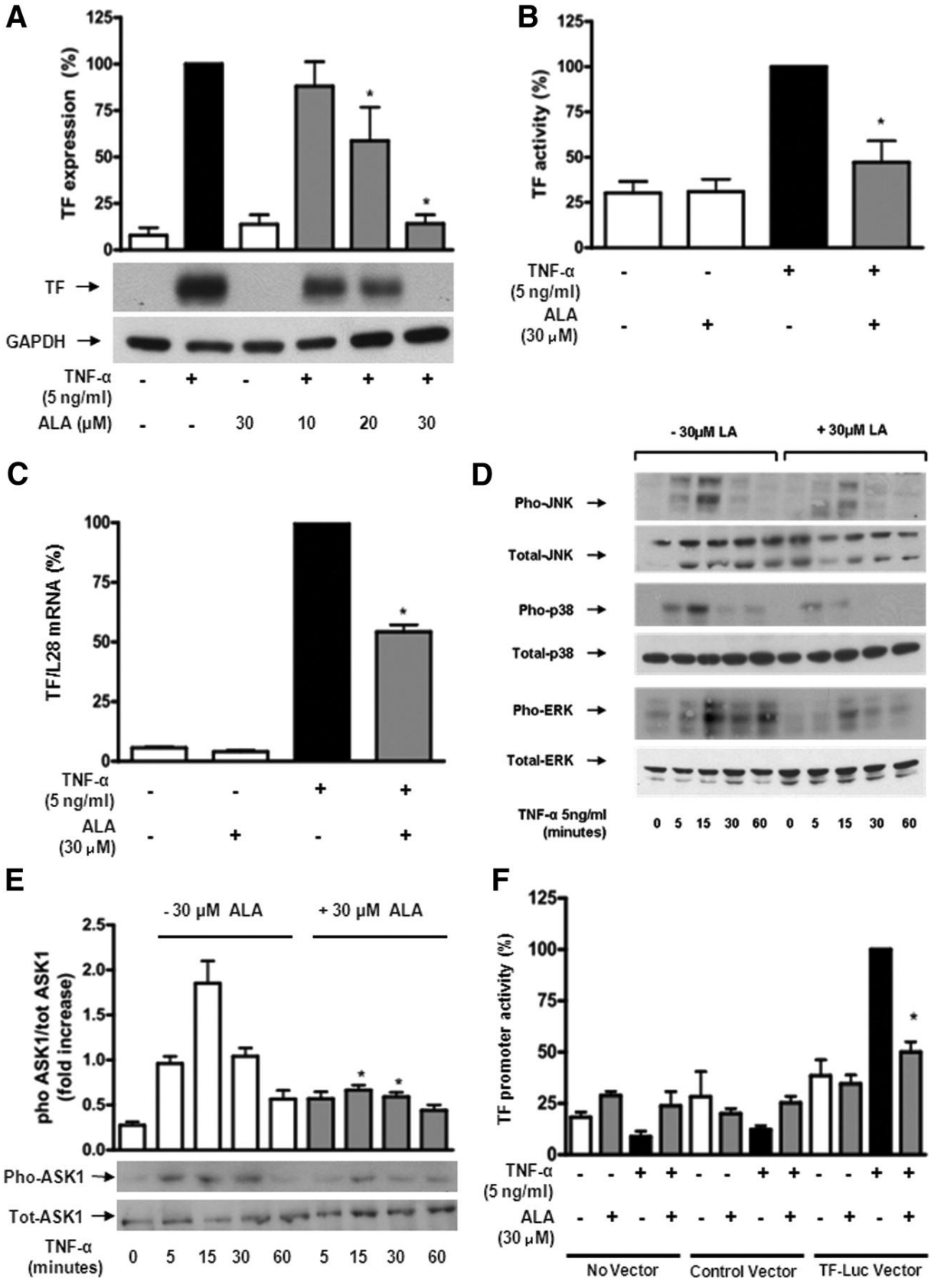

A
TF expression (%)
125
100
75
50
25
0
TF
GAPDH
TNF-α (5 ng/ml)
ALA (μM)
- + - + + +
- - 30 10 20 30
B
TF activity (%)
TNF-α (5 ng/ml)
ALA (30 μM)
C
TF/L28 mRNA (%)
D
- 30μM LA
+ 30μM LA
Pho-JNK
Total-JNK
Pho-p38
Total-p38
Pho-ERK
Total-ERK
TNF-α 5ng/ml (minutes)
0 5 15 30 60 0 5 15 30 60
E
- 30 μM ALA
+ 30 μM ALA
pho ASK1/tot ASK1 (fold increase)
Pho-ASK1
Tot-ASK1
TNF-α (minutes)
0 5 15 30 60 5 15 30 60
F
TF promoter activity (%)
No Vector
Control Vector
TF-Luc Vector

Figure 6

Figure Legends

Figure 1. Dietary ALA inhibits arterial thrombus formation.

A. Dietary ALA impairs time to thrombotic occlusion after photochemical injury in the mouse carotid artery *in vivo* (*p<0.005). **B**. Treatment with an inhibitory anti-TF antibody blunts the difference in occlusion times (p=NS). **C**. Sections of thrombosed carotid artery (L: vessel lumen, M: vessel media) from control and ALA treated animals (n=4) were stained for TF and fibrin (brown stain). Scale bars equal 50 μm; HE: hematoxylin and eosin stain. **D**. Sections from occluded carotid arteries were also stained for CD41 (red stain) and nuclei (blue stain). Scale bars equal 100 μm. HE: hematoxylin and eosin stain.

Figure 2. Dietary ALA inhibits platelet aggregation.

Thrombin (**A**) and collagen (**E**) induced platelet activation is inhibited in mice fed a high ALA diet as compared to control animals. Dietary ALA reduced maximal aggregation (**B**. thrombin: *p<0.005; **F**. collagen: *p<0.005), reduced area under the curve (**C**. thrombin: *p<0.005; **G**. collagen: *p<0.05), and increased lag time (**D**. thrombin: *p<0.05; **H**. collagen: p=NS).

Figure 3. Dietary ALA inhibits TF expression and NFκB activity in vivo.

Dietary ALA inhibits TF activity as assessed by FXa generation in carotid arteries (**A**. *p<0.005). **B**. Treatment of tissue lysates with an inhibitory anti-TF antibody (10 mg/ml) inhibits FXa generation (*p<0.01 versus control diet group in the presence of an IgG control antibody). Dietary ALA impairs TF mRNA expression (**C**. *p<0.05),

and NFkB p65 DNA binding affinity (**D**. *p<0.05) in mouse carotid arteries.

Figure 4. ALA inhibits TF expression in VSMC.

A. Treatment with ALA (30 μM) for 24h inhibits TF protein expression in VSMC (*p<0.05 versus control). **B**. ALA (30 μM) inhibits TF mRNA expression (*p<0.005 versus control) after 24h. **C.** ALA (30 μM) also impairs TF activity in VSMC (*p<0.001). **D**. Treatment of VSMC with an inhibitory anti-TF antibody inhibits FXa generation (*p<0.05 versus control treated with an IgG control antibody).

Figure 5. ALA inhibits p38 activation and NFkB activity in VSMC.

A. Treatment with ALA (30 μM) for 24h decreases p38 activation in VSMC (*p<0.05 versus control). **B**. Inhibition of p38 with SB203580 (10 μM) for 24h impairs TF expression in VSMC (*p<0.01 versus control). **C**. ALA (30 μM) reduces DNA binding activity of the NFκB subunit p65 in VSMC (*p<0.05 versus control).

Figure 6. ALA inhibits TNF-α induced TF expression in HAEC

A. Treatment with ALA (10-30 μM) for 1h inhibits TNF-α induced TF protein expression in human endothelial cells (*p<0.01 versus TNF-α alone). **B**. ALA (30 μM) inhibits TNF-α induced endothelial TF surface activity (*p<0.001 versus TNF-α alone). **C.** ALA (30 μM) inhibits TNF-α induced endothelial TF mRNA expression (*p<0.001 versus TNF-α alone). **D.** Incubation with ALA (30 μM) for 1h inhibits TNF-α induced transient phosphorylation of JNK, p38, and ERK. **E**. ALA (30 μM) inhibits TNF-α induced ASK1 phosphorylation (*p<0.005 versus TNF-α alone). **F.** ALA (30 μM) impairs endothelial TF promoter activation (*p<0.05 versus TNF-α alone).

Table I

Diet composition

	Low ALA		high ALA	
Composition (per kg)				
	g	**kcal**	**g**	**kcal**
DL-Methionine	3	12	3	12
Casein	195	780	195	780
Corn Starch	50	200	50	200
Maltodextrin 10	100	400	100	400
Sucrose	341	1364	341	1364
Cellulose, BW200	50	0	50	0
Corn Oil	10	90	10	90
Cocoa Butter	**137**	**1233**	**0**	**0**
Flaxseed Oil	**0**	**0**	**137**	**1233**
Primex Shortening	63	567	63	567
Mineral Mix S10001	35	0	35	0
Calcium Carbonate	4	0	4	0
Vitamin Mix V10001	10	40	10	40
	% (w/w)	**kcal%**	**% (w/w)**	**kcal%**
Protein	20	17	20	17
Carbohydrate	50	43	50	43
Fat	21	40	21	40
Total kcal/kg	**4676**		**4676**	
ALA (C18:3 *n*-3; % [w/w])	**0.03**		**7.3**	

Table II

Fatty acid profile in aortas from mice fed a low and high ALA diet

	low ALA (% of FA)	**high ALA** (% of FA)
SFA	**29.42**	**25.07**
MUFA	**59.21**	**40.47**
PUFA	**11.37**	**34.46**
Total n-3	**1.69**	**16.09**
n-6/n-3	**6.41**	**1.18**
ALA	**0.14**	**14.99**
EPA	**<0.1**	**<0.1**
DHA	**0.42**	**0.46**

SFA=saturated fatty acid; MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids; ALA=*alpha*-linolenic acid; EPA=eicasopentanoic acid; DHA=docosohexanoic acid

Table III

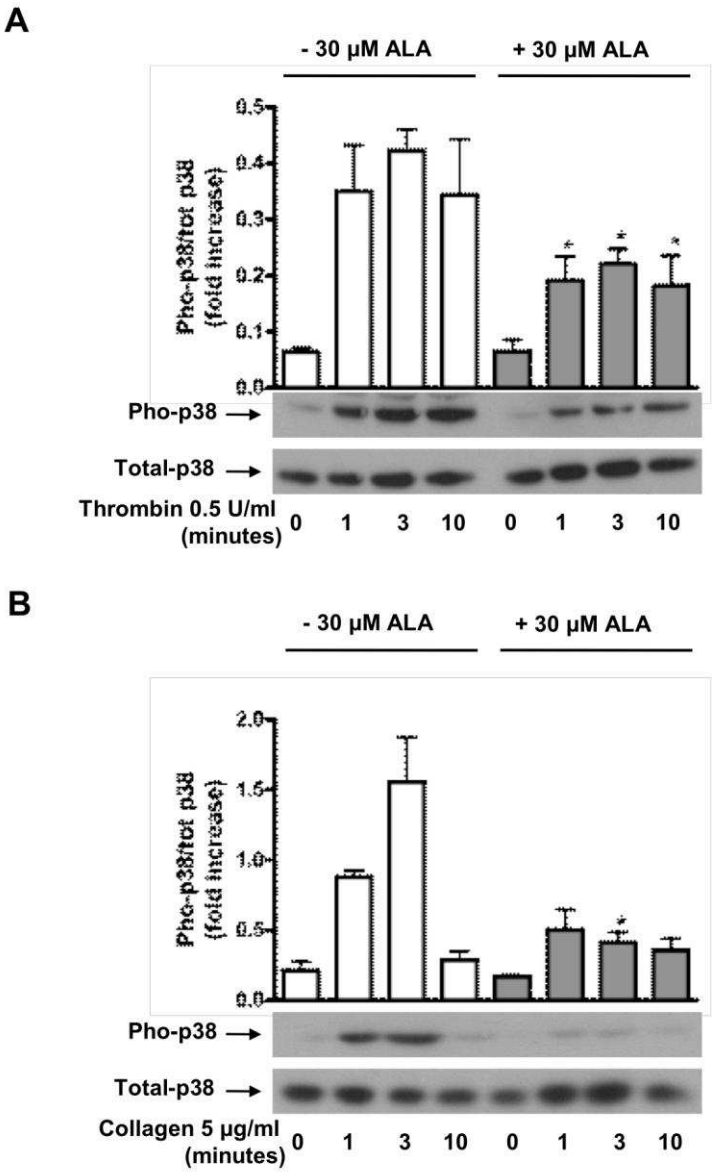
A. ALA inhibits thrombin induced human platelet activation ex vivo (n=5)

	Vehicle	30 µM ALA	*p*-value
Max. Aggregation (Ω)	28.25±1.7	17±1.8	<0.05
Area under the curve (Ωxmin)	104.4±9.8	53.3±9.3	<0.01
Lag time (seconds)	33.0±11.9	62.0±30.2	0.15

B. ALA inhibits collagen induced human platelet activation ex vivo (n=5)

	Vehicle	30 µM ALA	*p*-value
Max. Aggregation (Ω)	35.24±1.3	26±1.4	<0.005
Area under the curve (Ωxmin)	114.4±9.8	83.3±9.3	<0.01
Lag time (seconds)	18.0±4.9	34.0±4.2	<0.05

Figure I



Supplemental figure I. ALA inhibits thrombin and collagen induced p38 activation in platelets.

A. ALA (30 µM) abrogates thrombin induced p38 phosphorylation (*p<0.01 versus thrombin alone). **B**. ALA inhibits collagen induced p38 phosphorylation (*p<0.01 versus collagen alone).

Supplement Material

Material and methods

ALA diet and carotid artery thrombosis model

8-week-old male C57BL/6 mice weighing on average 24 g were fed a 0.21% w/w cholesterol diet containing either a high ALA (7.3% w/w , D06080702, Research Diets, New Brunswick, NJ, USA) or low ALA concentration (0.03% w/w, D06080701, Research Diets) for 2 weeks (supplemental table 1). ALA was supplemented as flaxseed oil in the high ALA group and replaced by cocoa butter in the control group. Thrombus formation was induced by photochemical injury on the 14^{th} day of diet. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (87 mg/kg; Butler, Columbus, OH). Rose bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted in phosphate-buffered saline (PBS) and then injected into the tail vein at a final concentration of 50 mg/kg. The right common carotid artery was exposed following a midline cervical incision and the blood flow monitored using a Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) connected to a flowmeter (Model T106, Transonic Systems). Photochemical injury was induced by a 1.5 mW green light laser (540 nm; Melles Griot, Carlsbad, CA) 6 minutes after intravenous rose bengal injection. From the onset of injury, blood flow was monitored until occlusion occurred, which was defined as a flow below 0.1 ml/min for at least 1 minute. Where mentioned, an inhibitory mouse anti-TF antibody obtained by immunization of rabbits against the extracellular murine TF amino acid domain 29-250 (American Diagnostica Inc., Stamford, CA, No. 4515,) was injected intravenously at a concentration of 3 mg/kg body weight 20 minutes prior to the rose bengal injection.

Immunohistochemistry

Occluded carotid arteries were harvested, fixed in 3% formalin and embedded in paraffin. Sections were stained for platelets (anti–mouse CD41; Abcam, Cambridge, UK; 1:50 dilution), fibrin (Anti-human, fibrin Fragment E, Cedarlane, Burlington, ON, Canada; 1:100 dilution) and TF (American Diagnostica; 1:50 dilution). Slides were blocked with 10% goat serum, and the primary antibody applied overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti–mouse (for fibrin and TF, 1:200 dilution), or Texas red conjugated goat anti-rat secondary antibodies (1:200 dilution). Slides were counterstained with hematoxylin and eosin (TF and fibrin) or 4',6-diamidino-2-phenylindole (CD41) .

Platelet preparation

For analysis of p38 expression, citrated whole blood was centrifuged at 170xg for 7 minutes to obtain platelet-rich plasma (PRP). PRP was centrifuged a second time at 170xg for 7 minutes to remove residual erythrocytes. To isolate platelets PRP was centrifuged at 350xg. Pelleted platelets were resuspended in tyrode buffer (10 mM Hepes, 12 mM $NaHCO_3$,137 mM NaCl, 2.7 mM KCl, 5 mM glucose; pH 7.4) and incubated with thrombin or collagen in the presence of ALA (30 µM) or the vehicle for various time points. Reactions were stopped by addition of ice-cold lysis buffer and p38 phosphorylation assessed by western blot analysis as described below.

Platelet count and aggregation

Platelets were counted by flow cytometry using whole blood collected in EDTA tubes (B&D Diagnostics, Franklin Lakes, NJ, USA). Platelet aggregation was studied using a Chrono-Log whole blood impedance aggregometer (Chrono-Log, Havertown, PA, USA). For studies performed in murine platelets, citrated blood was drawn by

puncture from the right ventricle. For studies in human platelets, blood was obtained from healthy human volunteers and treated with ALA (30 μM; Cayman Chemical, Ann Arbor, MI, USA) or vehicle (ethanol 0.1%, Sigma Aldrich, St. Louis, USA) for 30 minutes. Aggregation studies were performed with citrated blood within 1 hour. Platelets were equilibrated under constant stirring for 1 minute prior addition of human thrombin (0.5 U/mL; Sigma Aldrich) or equine collagen type 1 (5 µg/mL; Chrono-Log). Aggregation was displayed as a function of time (AGGRO/LINK® Software; Chrono-Log). Results were monitored for 6 minutes and expressed as maximal aggregation (ohm[Ω]), area under the curve (Ωxmin), and lag time (seconds).

Fatty acid profile in aortic tissue

Aortic tissue was pooled (n=7 per group) and FA profiles of the tissues were analyzed by gas chromatography. Fat was extracted via hexane:isopropanol (3:2) and triglycerides were saponified using methanolic sodium hydroxide. FA were converted to fatty acid methyl ester (FAME) with methanolic boron trifluoride. FAME were separated using a gas chromatograph (Hewlett Packard HP 6890 Series, GC Systems, Waldbronn, Germany) equipped with a 200 mm x 0,25 mm CP7421-column (Varian, Middleburg, NL). For FAME identification, a FAME mixture was used as external standard (Supelco 37 component FAME mix). The proportion of different FAME was calculated using the ratio of the peak area of the respective FAME to the sum of total FAME peak areas. FA were evaluated using the HP ChemStation software (Hewlett Packard, CA, USA). Measurements were performed in duplicate and the mean value displayed.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT)

Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500xg; 4°C; 15 minutes). PT and aPTT were assessed using the START 4 analyzer (Diagnostica Stago, France).

Thrombin generation

Plasma thrombin generation was monitored by automated calibrated thrombography.[1] Platelet poor plasma was mixed with human recombinant TF (5 pM) and a fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC Bachem Basle, Switzerland) and then recalcified. Thrombin generation was measured in a Fluoroskan® Ascent reader (Thermo Labsystems, Helsinki, Finland) and calculated using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). Endogenous thrombin potential, representing the area under the thrombin generation curve and time to peak thrombin formation (TTP) were displayed using the Prism 4 software package (GraphPad Software Inc., La Jolla, USA).

Cell culture

Human aortic vascular smooth muscle cells (VSMC; Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC; Clonetics) were cultured as described.[2] For experiments, VSMC were serum-starved for 24 hours before pretreatment with ALA (Cayman Chemical, Ann Arbor, MI, USA), stearic acid (SA, Sigma Aldrich), linoleic acid (LA, Sigma Aldrich) or vehicle (ethanol 0.1%, Sigma Aldrich) for 6 or 24 hours. To block the mitogen-activated protein (MAP) kinase p38 MAP kinase (p38), VSMC were treated with SB203580 (Sigma Aldrich; 10) for 24 hours. For experiments with HAEC, cells were incubated with ALA for 1 hour prior stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) for 4 hours for protein expression analysis and for 2h for analysis of TF mRNA expression. NFκB

activation was inhibited by ammonium pyrrolidinedithiocarbamate (PDTC) or BAY 11-7082 (both from Sigma Aldrich) pretreatment for 24 hours. To determine cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase release was applied (Roche, Basel, Switzerland).

Western blot

Protein expressionin was determined by Western blot analysis. Antibodies against human TF (No. 4503) and tissue factor pathway inhibitor (TFPI, No. 4901) (both from American Diagnostica, Stamford, CT) were used at 1:2500 dilution. Antibodies against phosphorylated p38, ERK, JNK, and ASK1 (all from Cell Signaling) were used at 1:1000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Antibodies against total p38, ERK, JNK, and ASK1 (all from Cell Signaling, Danvers, MA) were used at 1:2000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Blots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:10000 dilution, Chemicon International, Temecula, CA).

Factor Xa generation

To assess TF activity factor Xa generation on the surface of VSMC and HAEC was analyzed using a colorimetric assay (American Diagnostica Inc, ACTICHROME). TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. The absorbance of the reaction mixture was measured at 405 nm and values displayed after substraction of the background value. To confirm TF dependency of factor Xa generation in VSMC, cells were incubated with an inhibitory anti-TF antibody (American Diagnostica, No. 4509; 1:200) 30 minutes prior addition of exogenous factor VIIa.

For analysis of factor Xa generation in mouse carotid arteries, occluded right

carotid arteries from treated and control mice were homogenized in 50 µL lysis buffer (0.1% Triton-X, 100 mmol Nacl, 50 mmol Tris-Hcl, pH 7.4). 25 µg of tissue lysates were used for measurements. TF dependency of factor Xa generation in tissue lysates was confirmed by incubation with an inhibitory mouse anti-TF antibody (American Diagnostica; No. 4515; 10 mg/ml) 30 minutes prior addition of exogenous factor VIIa.

Real Time PCR

RNA was extracted from VSMC, HAEC and mouse aorta using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham, Buckinghamshire, UK). For real-time PCR analysis the following primers were used: for full length human TF (*F3*): sense 5'-TCCCCAGAGTTCACACCTTACC–3', antisense 5'–CCTTTCTCCTGGCCCATACAC–3'; for human ribosomal L28: sense 5'-GCATCTGCAATGGATGGT-3', antisense 5′-TGTTCTTGCGGATCATGTGT-3′; for murine full length tissue factor: sense: 5'-CAATGAATTCTCGATTGATGTGG-3', antisense: 5'-GGAGGATGATAAAGATGGTGGC-3'; for murine tissue factor pathway inhibitor: sense: 5'-ACTGTGTGTCTGTTGCTTAGCC-3', antisense: 5'-GTTCTCGTTCCCTTCACATCCC3'; and for murine ribosomal S12: sense: 5'-GAAGCTGCCAAAGCCTTAGA-3', antisense: 5'-AACTGCAACC-AACCACCTTC-3'. The amplification program consisted of 1 cycle at 95°C for 10 minutes followed by 35 cycles with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72° for 1 minute. Melting curve analysis confirmed the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for human TF and L28, a standard curve generated from serial dilutions of purified amplicons was

included. For murine TF and S12 the ΔΔCt threshold cycle method was used.

Nuclear Extracts and NFκB activity

For measurement of NFkB activity, VSMC were treated with ALA (30 μM) or the vehicle for 24 hours. Nuclear extracts were obtained from VSMC or mouse aorta using a nuclear extraction kit (Active Motif, Carlsbad, USA). Total protein (20 μg) was loaded in each well, and NFκB activity was measured using a TransAM NFκB p65 kit (Active Motif).

References

40. Tchaikovski SN, Van Vlijmen BJ, Rosing J, Tans G. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. J Thromb Haemost. 2007; 5:2079-2086.
41. Camici GG, Steffel J, Akhmedov A, Schafer N, Baldinger J, Schulz U, Shojaati K, Matter CM, Yang Z, Lüscher TF, Tanner FC. Dimethyl sulfoxide inhibits tissue factor expression, thrombus formation, and vascular smooth muscle cell activation: a potential treatment strategy for drug-eluting stents. Circulation. 2006; 114:1512-1521.